WORLD JOURNAL OF PHARMACEUTICAL RESEARCH
SJIF Impact Factor 7.523
Volume 6, Issue 4, 129-147. Research Article ISSN 2277– 7105

ANALYSIS OF PHYTOCHEMICAL, ANTIOXIDANT AND MICROBIAL PROPERTY OF VARIOUS EXTRACTS OF THE PLANT *MELILOTUS INDICA*

Jafrin Jobayer Sonju[1]*, MD. Fokhrul Islam[3], Kakan Sutradhar[2], Taslima Akter[4]

[1]Department of Pharmacy, Jahangirnagar University, Savar, Dhaka-1342, Bangladesh.
[2]Department of Pharmacy, Jahangirnagar University, Savar, Dhaka-1342, Bangladesh.
[3,4]Assistant Professor, Department of Pharmacy, Jahangirnagar University, Savar, Dhaka-1342, Bangladesh.

Article Received on
25 Jan. 2017,
Revised on 15 Feb. 2017,
Accepted on 08 March. 2017
DOI:10.20959/wjpr20174-8050

***Corresponding Author**
Jafrin Jobayer Sonju
Department of Pharmacy,
Jahangirnagar University,
Savar, Dhaka-1342,
Bangladesh.

ABSTRACT

Melilotus indica is a plant commonly known as 'Banmethi' in Bangladesh. It is a herb which has long been used traditionally for various medicinal purposes in all over the world. Being the member of Fabaceae family it claims a number of medicinal uses. In view of this, the present study was conducted to evaluate the phytochemical, antioxidant activity and microbial property of various extract of the leaf of the plant *Malilotus indica.* As evident from the phytochemical screening, leaf of *Melilotus indica* gave positive reaction for carbohydrates, glycosides (general test) and saponins. Methanolic and ethanolic extracts gave positive reactions for steroid. In case of alkaloid test, leaf of *Melilotus indica* gave positive reactions for mendroayer's reagent, hager's reagent, wagner's reagent and dragdroff's reagent for both ethanoic and chloroformic extracts. The antioxidant activity of the different extracts of *Melilotus indica* was evaluated using different methods like total phenol content determination, determination of flavonoid content, determination of total anitioxident capacity and DPPH radical scavenging assay. The extracts were found to posses moderate amount of phenolics, flavonoids and total antioxidant capacity expressed as gallic acid equivalent(GAE), quercetin equivalent(QE) and ascorbic acid(AAE) equivalent respectively. In DPPH radical scavenging assays, the crude extract of *Melilotus indica* leaf showed dose dependent scavenging of DPPH radicals in a way similar to that of the reference antioxidant ascorbic acid. In antimicrobial activity test, the antimicrobial screening of methanolic extract

of *Melilotus indica* leaf shows sensitivity to gram positive organism *Staphylococcus aureus* but shows resistance to gram negative organism *Escherichia coli.* The result of the investigation of the present study lend partial scientific support in favor of the traditional use of *Melilotus indica,* while traditional use varies among the local practitioners and scientific evidence that support the traditional uses is not well established yet**.** Thus the plant deserve extensive and well-designed research on the basis of the current study results. It will help to find bioactive compounds as new lead compounds which could be responsible for their antioxidant and microbial actions.

KEYWORDS: *Melilotus indica*, phytochemical screening, antioxidant activity, property.

INTRODUCTION

Plants that possess therapeutic properties or beneficial pharmacological effects on animal body are generally classified as Medicinal plants. These medicinal plants have been used in traditional folk medication as efficacious remedies for hundreds of years. In Bangladesh, more than 1000 floral species out of 5000 are possibly used in the various practices of traditional medication. The indigenous medicinal plants utilized have been increasing in number, still today, with the discovery and introduction of newer plants. Today, in the traditional medication system, nearly every plants and herbs growing in the country have been assumed that they carry some medicinal virtues, and that are used in the preparation of medicine either as principle therapeutic agent or necessary associate (recipient) to increase the potency of the main agent as well as to make it more stable. The estimate says that more than 1000 metric tons of medicinal plants are required by the industries involved in the manufacture of traditional medication in Bangladesh.

However, the medicinal efficacies of plants' properties used for the traditional medication have not been always substantiated scientifically, providing proper chemical data and botanical facts of the constituents of each plant. To prove scientifically and academically that the plants used for the traditional medication to have the proper properties as medicinal plants, it is indispensable to carry on academic and experimental researches correctly and attentively, presenting all the scientific date and details of each plant with unerring precision.[1]

Plant Information

Scientific Name

Melilotus indicus (L.) All.

Synonym

Melilotus indica

Common Names

Annual Yellow Sweet Clover, Banmethi, Indian Sweet-clover, Sour clover, The small flowers are in narrow, elongated racemes. The individual flowers are pea like and only 3 mm long. The leaves are green, alternate, and have 3 sharply-toothed, oblanceolate leaflets with blunt or indented tips. Annual or biennial, erect or prostrate herb, branching from base, 10-50cm high sometimes to 80-100cm in favorable conditions.The similar Yellow Sweetclover (*Melilotus officinalis*) has point-tipped leaflets and larger flowers that are usually twice as long.[2]

Figure: 1 *Melilotus indica*.

The plant is antispasmodic, emollient, analgesic, insect repellent, tonic, astringent; it is used for swellings, tumors, skin rash, wounds, gastrointestinal problems and colds. Seeds taken to treat genital organ diseases.

In surface view, the upper epidermis is shown to consist of polygonal cells that have thick and more or less sinuous beady cell walls. A transverse section of the leaf lamina shows the leaf to be dorsiventral with the palisade tissues exclusively underlying the upper epidermis.

The oval stomata are numerous and they are of the anomocytic type but the anisocytic type of stomata is also observed. The upper epidermis is covered with a thin unstriated cuticle.

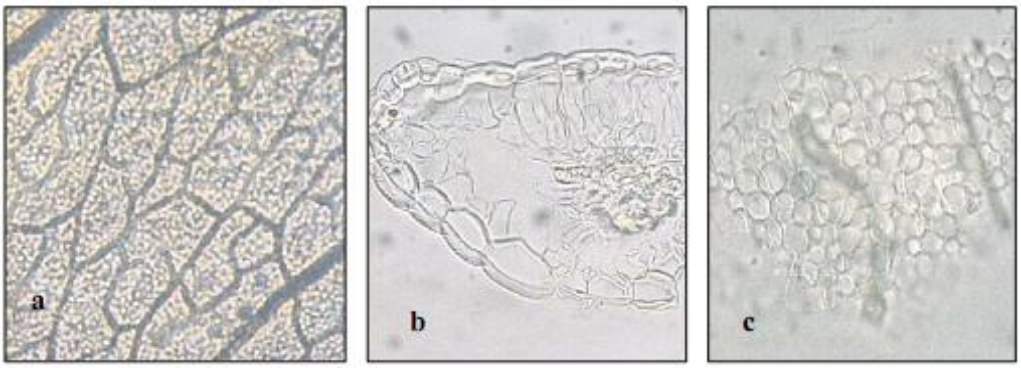

Figure: 2 (a). A general surface view of the lower epidermis of the leaf showing the intricatetype of venation. (b). TS of the leaf near the margin showing the oblong upper epidermal cells and the relatively large lower epidermal cells, the palisade tissues and the spongy mesophyll cells embedding vascular tissues. (c). A portion of the leafshowing the comparatively smaller upper epidermal cells (including oval stomata)[3]

Coumarin, herniarin, umbelliferene and scopoletin have been identified in the plant. The presence of β-sitosterol, a sterol or triterpene alcohol, choline and an aromatic compound have also been reported.[4] *Melilotus indica* has been reported to be emollient, astringent, strongly laxative and narcotic.[3] The plant contains coumarin, which is reported to be anticoagulant (Duke & Ayensu 1985). The dried leaves are reported to be toxic, whereas the fresh leaves are quite safe. This is due to the presence of coumarin, the substance that gives some dried plants the smell of hay. If taken internally it can prevent the blood from clotting[3] the leaves and the aerial parts of the plant are known to as insect repellent (Moerman, 1998).[5]

MATERIAL AND METHODS

At first with the help of a comprehensive literature review plant *Melilotus indica* of the family Fabaceae was selected for this investigation. The whole plants was collected from Dhaka, Bangladesh and identified by the taxonomist of the National Herbarium of Bangladesh, Mirpur, Dhaka. The voucher specimens of the plants have been deposited in the herbarium for further reference.

The leaves of the plants were collected in fresh condition. It was sun-dried and then, dried in an oven at reduced temperature (not more than 50 ^{0}C) to make suitable for grinding purpose.

The coarse powders were then stored in air-tight container with necessary markings for identification and kept in cool, dark and dry place for the investigation. The powdered plant materials were submerged into ethanol in an air-tight flat bottomed container for seven days, with occasional shaking and stirring. The major portion of the extractable compounds of the plant materials were dissolved in the solvent.

In this research work methanolic, ehtanolic, aqueous and chloroformic extracts of the leaf of *Melilotus indica*were screened for carbohydrates, glycosides (general as well as anthraquinone), saponins, flavanoids, tannins, steroids, alkaloids that have pronounced medical value.Small amount of dried, decolorized extracts were appropriately treated to prepare sample solution and then subjected to various phytochemical tests.

Various phytochemical tests which were performed under heading of phytochemical screening are mentioned below:

Molisch's test for carbohydrates

Two drops of molisch's reagents were added to about 5 mg of the extract in 5 ml aqueous solution in a test tube. 1 ml of conc. H_2SO_4 was allowed to flow down the side of the inclined test tube so that the acid formed a layer beneath the aqueous solution without mixing with in. a red ring was formed at the common surface of the two liquids which indicated the presence of carbohydrate. On standing or shaking a dark-purple solution was formed. Then the mixture was shaken and diluted with 5 ml of water. Dull violet precipitate was formed immediately.

General test for glycosides

A small amount of extract was dissolved in 1ml of water then few drops of aqueous NaOH solution was added. A yellow color was developed in the presence of glycosides.

Test for glycosides

A small amount of extract was dissolved in water and alcohol then boiled with Fehling's solution. Any brick-red precipitation was noted. Another portion of extract was dissolved in water and alcohol and boiled with a few drops of dilute H_2SO_4. The acid was neutralized with NaOH solution and boiled with Fehling's solution. A brick-red precipitation was produced in this experiment which showed the presence of glycosides in the extract.

Borntragers's test for anthraquinone glycosides

1 ml of sample solution was shaken with 5 ml of chloroform in a test tube for at least 5 minutes then again shaken with an equal volume of 10% ammonia solution. A bright pink, red or violet color was developed in the aqueous (upper) layer in the presence of free anthraquinones.

Tests for alkaloid

A small volume of each extract was neutralized by adding 1 or 2 drops of dilute H_2SO_4 . This neutralized solution was treated with a very small amount of the following reagents and the respective color and precipitate formation was observed:

- **Mayer's reagent:** Formation of white and cream color precipitate indicated the presence of alkaloids.
- **Hager's reagent**: Formation of yellow crystalline precipitate indicated the presence of alkaloids.
- **Wagner's reagent**: Formation of brownish-black ppt indicated the presence of alkaloids.
- **Dragendroff's reagent:** Formation of orange or orange-red precipitate indicated the presence of alkaloids.

Test for saponins

About 0.5 ml of extract was shaken vigorously with water in a test tube. If a forthing was produced and it was stable for 1-2 minutes and persisted on warming, it was taken as preliminary evidence for the presence of saponins.

Test for flavonoids

A few drops of conc. HCl was added to a small amount of an extract. Immediate development of a red color indicated the presence of flavanoid.

Test for steroids

A small amount of extract was added with 2 ml of chloroform, then 1 ml of conc. H_2SO_4 was carefully added from the side of the test tube. In presence of steroids ,a red color was produced in chloroform layer.

Test for tannins

About 0.5 ml of extract was stirred with 10 ml of distilled water. Production of a blue, blue-black, green or blue-green coloration or precipitation on the addition of $FeCl_3$ (5%) reagent was taken as evidence for the presence of tannins.

In vitro determination of the antioxidant activities

Majority of the diseases/disorders are mainly linked to oxidative stress due to free radicals. Free radicals are fundamental to any biochemical process and represent an essential part of aerobic life and metabolism. The most common reactive oxygen species (ROS) include superoxide (0_2.) anion, hydrogenperoxide (H_20_2), peroxyl (ROO-) radicals, and reactive hydroxyl (OH.) radicals. The nitrogen derived free radicals are nitric oxide (NO.) and peroxynitrite anion(ONOO.). ROS have been implicated in over a hundreds of diseases states which range from arthritis and connective tissue disorders to carcinogenesis, aging, physical injury, infection and acquired immunodeficiency syndrome in treatment of these diseases, antioxidant therapy has gained an immense importance. [6] Current research is now directed towards finding naturally occurring antioxidants of plant origin. Antioxidants have been reported to prevent oxidative damage by free radical and ROS, and may prevent the occurrence of disease, cancer and aging. It can interfere with the oxidation process by reacting with free radicals, chelating, catalytic metals, and also by acting as oxygen scavengers. Plant and plant products are being used as a source of medicine since long. The medicinal properties of plants have been investigated in the recent scientific developments throughout the world, due to their potent antioxidant activities, no side effects and economic viability. Flavonoids and phenolic compounds widely distributed in plants which have been reported to exert multiple biological effect, including antioxidant, free radical scavenging abilities, anti-inflammatory, anticarcinogenic etc. They were also suggested to be a potential iron chelator. Novel natural antioxidants from some plants have been extensively studied in the past few years for their antioxidant and radical scavenging properties.

The antioxidant activity of the methanolic, ehtanolic, aqueous and chloroformic extracts of the leaf of *Melilotus indica*were determined by using the following methods.

- Determination of Total Phenol
- Determination of Flavonoid Content
- Determination of Total Antioxidant Capacity
- DPPH (1,1-diphenyl-2-picrylhydrazyl) Scavenging capacity Assay[7]

Determination of Total Phenol

The content of total phenolic compounds in plant ethanolic extracts were determined by Folin–Ciocalteu Reagent (FCR). The FCR actually measures a sample's reducing capacity. The exact chemical nature of the FC reagent is not known, but it is believed to contain heteropolyphosphotunstates - molybdates. Sequences of reversible one- or two-electron reduction reactions lead to blue species, possibly $(PMoW_{11}O_{40})^{4-}$. In essence, it is believed that the molybdenum is easier to be reduced in the complex and electron-transfer reaction occurs between reductants and Mo(VI):

$$Mo(VI) + e \rightarrow Mo\ (V)$$

Each plant extracts of weight 0.050 gm were taken and dissolved into 5 ml of methanol. The concentration of this solution was 10µg/µl of plant extract.

1.0 ml of each plant extracts or standard of different concentration solution were taken in test tubes and 5 ml of Folin–ciocalteu (Diluted 10 fold) reagent solution was added to the test tubes. 4 ml of Sodium carbonate solution was added into the test tubes. The test tubes were incubated for 30 minutes at 20^0C to complete the reaction (only for standard). The test tube was incubated for 1 hour at 20^0C to complete the reaction (only for extract).The absorbances of the solutions were measured at 765 nm using a spectrophotometer against blank. The Total content of phenolic compounds in plant methanol extracts in gallic acid equivalents (GAE) were calculated by the following formula equation

$$C = (c \times V)/m$$

Where

C = total content of phenolic compounds, mg/g plant extract, in GAE;

c = the concentration of gallic acid established from the calibration curve, mg/ml;

V = the volume of extract, ml;

m = the weight of pure plant methanolic extract, g.

Determination of flavonoid content[8]

Aluminum chloride colorimetric method was used for flavonoids determination.1 ml of sample was mixed with 3 ml of methanol, 0.2 ml of 10% aluminum chloride, 0.2ml of 1 M potassium acetate and 5.6 ml of distilled water. It remained at room temperature for 30 min; the absorbance of the reaction mixture was measured at 415 nm with UV/Visible spectrophotometer. The calibration curve was prepared by preparing quercetin solutions at

various concentrations in methanol. The concentration of flavonoids was expressed in terms of mg/100ml of sample.

10 gm of $AlCl_3$ was taken into a 100 ml of a volumetric flask and the volume was adjusted by distilled water. 9.815 gm of potassium was taken into a 100 ml of a volumetric flask and the volume was adjusted by distilled water. 1.0 ml of each plant extracts or standard of different concentration solutions were taken in different test tubes and 3 ml of methanol were added into the test tubes. 200µl of 10% aluminum chloride solution, 200µl of 1M potassium acetate solution and 5.6 ml of distilled water were added into the test tubes. The test tubes were incubated for 30 minutes at room temperature to complete the reaction. The absorbances of the solutions were measured at 415 nm using a spectrophotometer against blank. The Total content of flavonoid compounds in plant methanol extracts in quercetin equivalents was calculated by the following formula equation

$$C = (c \times V)/m$$

Where

C = total content of flavonoid compounds, mg/g plant extract, in quercetin;

c = the concentration of quercetin established from the calibration curve, mg/ml;

V = the volume of extract, ml;

m = the weight of pure plant methanolic extract, g.

Determination of Total Antioxidant Capacity

The phosphomolybdenum method usually detects antioxidants such as ascorbic acid, some phenolics, α-tocopherol, and carotenoids. The phosphomolybdenum method was based on the reduction of Mo(VI) to Mo(V) by the antioxidant compound and subsequent formation of a green phosphate/Mo(V) complex at acid pH.. In essence, it is believed that the molybdenum is easier to be reduced in the complex and electron-transfer reaction occurs between reductants and Mo(VI) and the formation of a green phosphate/Mo(V) complex with a maximal absorption at 695 nm.

$$Mo(VI) + e \rightarrow Mo\ (V)$$

300µl of each plant extracts or standard of different concentration solutions were taken into different test tubes and 3 ml of reagent solution was added into each of the test tubes.The test tubes were incubated at 95^0C for 90 minutes to complete the reaction. The absorbances of the solutions were measured at 695 nm using a spectrophotometer against blank after cooling to room temperature. A typical blank solution contained 3 ml of reagent solution and the

appropriate volume (300µl) of the same solvent used for the sample and incubated under the same conditions as the rest of the samples solution. The antioxidant activity is expressed as the number of equivalents of ascorbic acid and was calculated by the following formula equation

$$A = (c \times V)/m$$

Where

A = total content of Antioxidant compounds, mg/g plant extract, in Ascorbic acid;

c = the concentration of Ascorbic acid established from the calibration curve, mg/ml;

V = the volume of extract, ml;

m = the weight of pure plant methanolic extract, g.

DPPH (1, 1-diphenyl-2-picrylhydrazyl) Scavenging capacity Assay

The 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) has been widely used to evaluate the free radical scavenging capacity of antioxidants. DPPH free radical is reduced to the corresponding hydrazine when it reacts with hydrogen donors. DPPH can make stable free radicals in aqueous or methanol solution. With this method it was possible to determine the antiradical power of an antioxidant activity by measurement of the decrease in the absorbance of DPPH at 517 nm. Resulting from a color change from purple to yellow the absorbance decreased when the DPPH was scavenged by an antioxidant, through donation of hydrogen to form a stable DPPH molecule. In the radical form this molecule had an absorbance at 517 nm which disappeared after acceptance of an electron or hydrogen radical from an antioxidant compound to become a stable diamagnetic molecule.

200µl of each plant extracts or standard of different concentration solutions were taken in different test tubes and 2 ml of reagent solution was given into each test tube. The test tubes were incubated for 30 minutes to complete the reaction. The absorbance of the solution was measured at 517 nm using a spectrophotometer against blank. A typical blank solution contained ethanol or methanol. The percentage (%) inhibition activity was calculated from the following equation

$$(A_o - A_1)/A_o\} \text{ X } 100$$

Where,

A_0 is the absorbance of the control, and

A_1 is the absorbance of the extract/standard.

Then % inhibitions were plotted against log concentration and from the graph IC_{50} was calculated.[9-10]

Antimicrobial screening

Solutions of known concentration (µg/ml) of the test samples are made by dissolving measured amount of the samples in calculated volume of solvents. Dried and sterilized filter paper discs (6 mm diameter) are then impregnated with known amounts of the test substances using micropipette. Discs containing the test material are placed on nutrient agar medium uniformly seeded with the test microorganisms. Standard antibiotic discs and blank discs (impregnated with solvents) are used as positive and negative control. These plates are then kept at low temperature (4^0C) for 24 hours to allow maximum diffusion. During this time dried discs absorb water from the surrounding media and then the test materials are dissolved and diffused out of the sample disc. The diffusion occurs according to the physical law that controls the diffusion of molecules through agar gel.[11] As a result there is a gradual change of test materials concentration in the media surrounding the discs.

The plates are then incubated at 37^0C for 24 hours to allow maximum growth of the organisms. If the test materials have any antimicrobial activity, it will inhibit the growth of the microorganisms and a clear, distinct zone of inhibition will be visualized surrounding the medium. The antimicrobial activity of the test agent is determined by measuring the diameter of zone of inhibition expressed in millimeter. In the present study the crude methanolic, ehtanolic, aqueous and chloroformic extracts of the leaf of *Melilotus indica* were tested for antimicrobial activity by disc diffusion method.

The bacterial strains used for the experiment were collected as pure cultures from the Institute of Nutrition and Food Science (INFS), University of Dhaka. Both Gram positive and Gram-negative organisms were taken for the test and they are listed in the Table.

Table: 1 List of test bacteria

Gram positive Bacteria	Gram negative Bacteria
Staphylococcus aureus	*Escherichia coli*

To prepare required volume of this medium, calculated amount of each of the constituents was taken in a conical flask and distilled water was added to it to make the required volume. The contents were heated in a water bath to make a clear solution. The P^H (at 25 ^{0}C) was

adjusted at 7.2 – 7.6 using NaOH or HCl. 10 ml and 5 ml of the medium was then transferred in screw cap test tubes to prepare plates and slants respectively. The test tubes were then capped and sterilized by autoclaving at 15-lbs. pressure/ sq. inch at 121 ^{0}C for 20 minutes. The slants were used for making fresh culture of bacteria and fungi that were in turn used for sensitivity study.

In an aseptic condition under laminar air hood cabinet, the test organisms were transferred from the pure cultures to the agar slants with the help of a transfer loop to have fresh pure cultures. The inoculated strains were then incubated for 24 hours at 37 ^{0}C for their optimum growth. These fresh cultures were used for the sensitivity test. [12] The test organisms were transferred from the subculture to the test tubes containing about 10 ml of melted and sterilized agar medium with the help of a sterilized transfer loop in an aseptic area. The test tubes were shaken by rotation to get a uniform suspension of the organisms. The bacterial and fungal suspension was immediately transferred to the sterilized petridishes. The petridishes were rotated several times clockwise and anticlockwise to assure homogenous distribution of the test organisms in the media.

These were used as positive control to ensure the activity of standard antibiotic against the test organisms as well as for comparison of the response produced by the known antimicrobial agent with that of the test sample. In this investigation, kanamycin (30µg/disc) standard disc was used as the reference. These were used as negative controls which ensure that the residual solvent (left over the discs even after air-drying) and the filter paper were not active themselves.

Sample discs were prepared by adding 20 µl of the test solutions to the sterile filter paper discs. The discs were then allowed to dry for sufficient period of time until complete evaporation of the solvent. The test samples were applied to previously sterilized discs using adjustable micropipette under aseptic conditions. The sample discs, the standard antibiotic discs and the control discs were placed gently on the previously marked zones in the agar plates pre-inoculated with test bacteria. The plates were then kept in a refrigerator at 4 ^{0}C for about 24 hours upside down to allow sufficient diffusion of the materials from the discs to the surrounding agar medium. The plates were then inverted and kept in an incubator at 37^{0}C for 24 hours.

The antimicrobial potency of the test agents are measured by their activity to prevent the growth of the microorganisms surrounding the discs which gives clear zone of inhibition. After incubation, the antimicrobial activities of the test materials were determined by measuring the diameter of the zones of inhibition in millimeter with a transparent scale.[13]

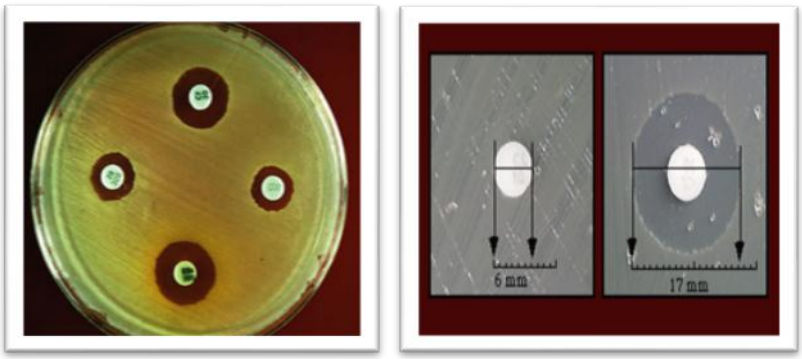

Figure - 3: Measuring zone of inhibition

RESULT

Since the chemical constituents present in a plant are directly responsible for its therapeutic and other pharmacological properties, the constituents of the plant which are detected during this investigation should have some direct relationship with local medicinal uses.

Preliminary phytochemical screening of the Leaf of *Melilotus indica* revealed the presence of different kind of chemical groups that are summarized in Table 2 & 3.

Table: 2: Phytochemical screening of *Melilotus indica* leaf extract.

Tests	Methanolic extract of Leaf	Ethanolic extract of Leaf	Chloroformic extract of Leaf	Aqueous extract of Leaf
1. Carbohydrates	+	+	+	+
2. Glycosides	+	+	+	+
3. Saponins	+	+	-	+
4. Flavonoids	-	-	-	-
5. Steroids	+	+	-	-
6. Tannins	-	-	-	-
7. Anthraquinone glycosides (Borntragers's test)	-	-	-	-

Table: 3: Test results of alkaloid phytochemical screening

Reagent Name	Methanolic extract of Leaf	Ethanolic extract of Leaf	Chloroformic extract of Leaf	Aqueous extract of Leaf
Mayer's Test	-	+	+	-
Hager's Test	+	+	+	-
Wagner test	-	+	+	-
Dragendorff's Test	+	+	+	-

[Here, '+' stands for the Presence, '-' stands for the Absence]

As evident from the above tables (Table 2 & 3), all the extracts of the leaf of *Melilotus indica*gave positive reaction for carbohydrate, glycoside (General test), saponins and alkaloids tests.Only methanolic extract of the leaf shows positive reaction to steroids. In aqueous extract of the leaf shows negative reactions to Mayer's Test, Hager's Test, Wagner test, Dragendorff's Test and methanolic extract of the leaf shows negative reactions to Mayer's Test & Dragendorff's Test.

Antioxidant Activity

Total Phenol Content Determination

Total phenolic content of the leaf extract of *Melilotus indica* was determined by using the Folin-Ciocalteu reagent and were expressed as Gallic acid equivalents (GAE) per gram of plant extract. The total phenolic contents of the test fractions were calculated using the standard curve of Gallic acid (y= 0.009x + 0.058; R^2 = 0.999) (Figure 4). Methanolic extract of leaf showed highest amount of phenolic contents among the other three extracts. Aqueous extract of leaf showed small amount of presence of phenol contents in comparison with the standerd Galic acid (Table - 4).

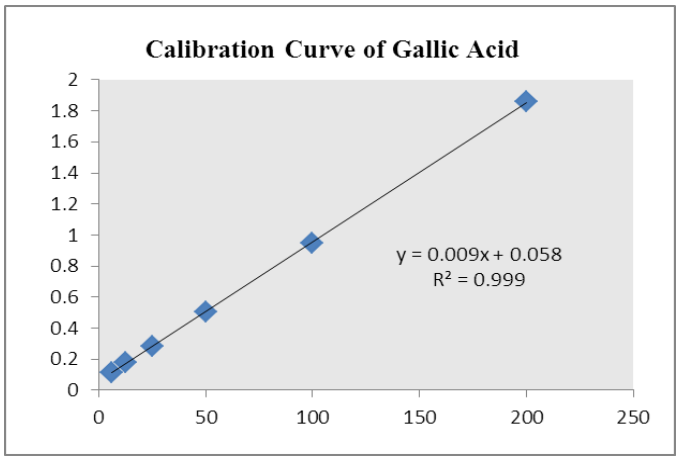

Figure-4: Calibration curve of gallic acid

Table: 4 Total phenol contents of the leaf of *Melilotus indica*

Sample	Total phenols mg/g plant extract (Galic Acid Equivalent)
Methanolic extract of Leaf	2.83
Ethanolic extract of Leaf	2.57
Chloroformic extract of Leaf	2.38
Aqueous extract of Leaf	1.66

The order of the total phenolic content of various extracts of leaf follows following order:

Methanolic extract > Ethanolic extract > Chloroformic extract > Aqueous extract

Determination of Total Flavonoid Content

Aluminium chloride colorimetric method was used to determine the total flavonoid contents of various extracts of leaf of *Melilotus indica*The total flavonoid content was calculated using the standard curve of quercetin (y = 0.005x - 0.005; R^2 = 0.996) (Figure 5) and was expressed as quercetin equivalents (QE) per gram of the plant extract. Ethanol extract of leaf of *Melilotus indica* was found to contain less amount of flavonoid. (Table - 5).

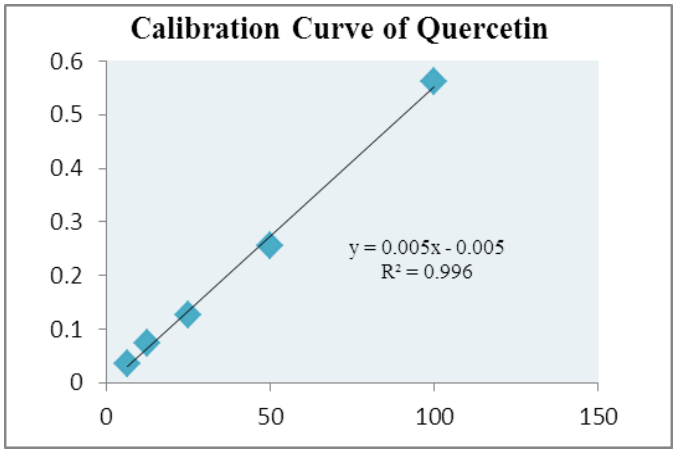

Figure: 5 Calibration curve of quercetin

Table: 5 Total Flavonoid contents of various extracts of leaf *Melilotus indica*.

Sample	Total flavonoid contents mg/g plant extract (Quercetin Equivalent)
Methanolic extract of Leaf	0.99
Ethanolic extract of Leaf	1.16
Chloroformic extract of Leaf	0.63
Aqueous extract of Leaf	0 .18

The order of the total phenolic content of various extracts of leaf follows following order:

Ethanolic extract > Methanolic extract > Chloroformic extract > Aqueous extract

Total Antioxidant Capacity

Total antioxidant capacity of the various extracts of leaf of *Melilotus indica*was evaluated by the phosphomolybdenum method and was expressed as ascorbic acid equivalents (AAE) per gram of plant extract. Total antioxidant capacity of the test samples was calculated using the standard curve of ascorbic acid (y = 0.006x + 0.101; R^2= 0.991) (Figure 6). Various extract of leaf of *Piper betle* was found to be potent in this test (Table 6).

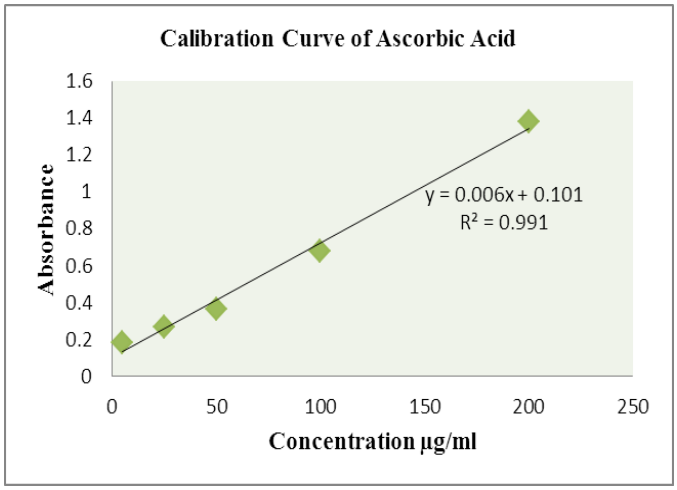

Figure: 6 Calibration curve of ascorbic acid

Table: 6 Total antioxidant capacity of the various extracts of leaf of *Melilotus indica*.

Sample	Total Antioxidant Capacity mg/g plant extract (Quercetin Equivalent)
Methanolic extract of Leaf	4.87
Ethanolic extract of Leaf	5.89
Chloroformic extract of Leaf	4.92
Aqueous extract of Leaf	4.46

The order of the Total antioxidant capacity of various extracts of leaf follows following order:

Ethanolic extract > Chloroformic extract > Methanolic extract > Aqueous extract

The phosphomolebdnum method was based on the reduction of Mo(VI)to Mo(V) by the antioxidant compound and the formation of a green phosphate/Mo(V) complex with a maximal absorption at 696 nm.

DPPH radical scavenging assay

When DPPH accepts an electron donated by an antioxidant compound, the DPPH is decolorised, which can be quantitatively measured from the changes in absorbance at 517 nm. The IC_{50} values of methanol extract of *Melilotus indica leaf* is presented in the Table 7. % scavenging of DPPH radical was found to decrease with increasing concentration of the extract.

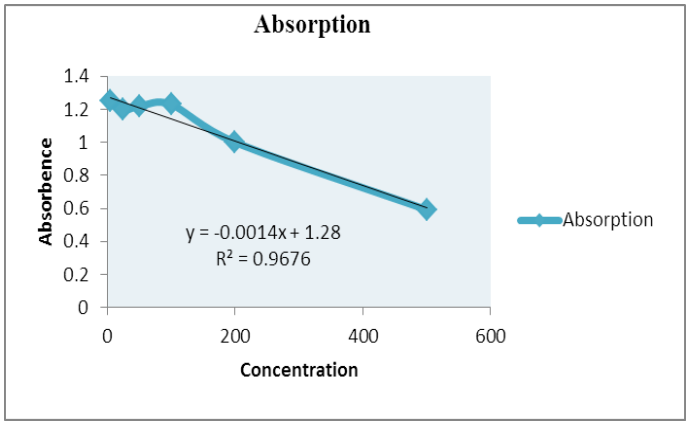

Figure: 7 Calibration curve of DPPH

Table: 7 IC_{50} values of methanol extract of *Melilotus indica*.in DPPH scavenging assay

Sample/Standard	IC_{50} (mg/ml)
Leaf(methanol)	0.854

In DPPH radical scavenging assays, the crude extract of *Melilotus indica*leaf showed dose dependent scavenging of DPPH radicals in a way similar to that of the reference antioxidant ascorbic acid. DPPH radical scavenging is a popular and reliable method for screening the free radical scavenging activity of compounds or antioxidant capacity of plant extracts. The DPPH antioxidant assay is based on the ability of 1, 1-diphenyl-2-picrylhydrazyl (DPPH), a stable free radical, to decolorize in the presence of antioxidants. The DPPH radical contains an odd electron, which is responsible for the absorbance at 515-517 nm and also for a visible deep purple color. When DPPH accepts an electron donated by an antioxidant compound, the DPPH is decolorized, which can be quantitatively measured from the changes in absorbance. Extract of *Melilotus indica* leaf has the ability to donate electron. (Table 7).

Antimicrobial Activity Determination

The result of antimicrobial screening of *Melilotus indica*leaf *is* given below in a table. Chloramphenicol is used as standard.

Table -8: List of bacteria with Inhibition Zone

Test Organisms	**Inhibition Zone Diameter (mm)**		
	Rifampicin	**Concentration of leaf extract, mg**	**Leaf extract**
Escherichia coli (Gram+)	Resistent	200	Resistent
		100	Resistent
		50	Resistent
Staphylococcus aureus (Gram-)	35	200	25
		100	15
		50	07

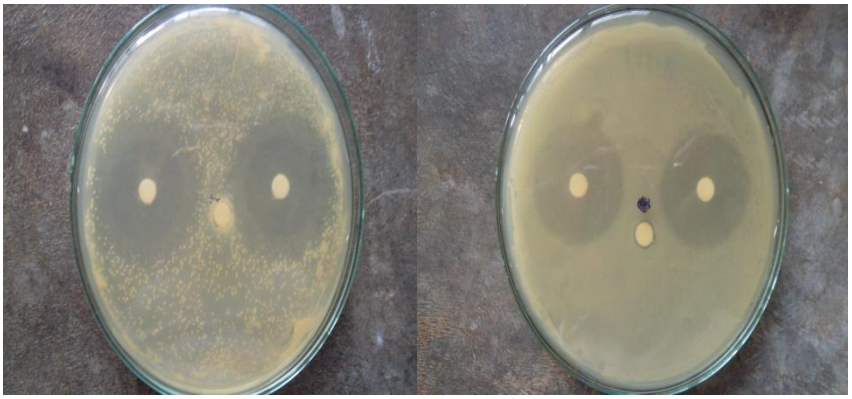

Figure 4.5: Zone of inhibition

Leaf extract of *Melilotus indica* exhibited antimicrobial activity only against one bacteria and it's probably attributed to the presence of steroids and saponin. [14]

CONCLUSION

This study showed that the plant leaf extract of *Melilotus indica* exhibits antioxidant activity as well as antimicrobial activity to gram negative organism like *Staphylococcus aureus*. The phytochemical investigations confirm the presence of steroids and saponins which plays role in antimicrobial activity. The values of absorbance of the leaf extract in UV spectroscopy vary between different extracts because of the solvent effects. The purpose of using different solvents is to find and compare the extraction capacity of the solvents and to measure the effects in analysis of those extractions. It shows different values in analysis for various solvents and the proficiency of results also varies. *Melilotus indica* is a herb of Fabacea family which needs further investigations to discover its pharmacological and medical use.

ACKNOWLEDGMENTS

The authors are grateful to department of pharmacy of Jahangirnagar University for providing facilities and support in this research.

REFERENCES

1. Mia, A.W. & Ghani, A., 1990, In: Ghani, A. (ed.), Traditional Medicine, Jahangirnagar University, Savar, Dhaka, Pages 10, 12.
2. Alami, R.& Macksad, A.Medicinal Plants of Kuwait, Al-assriya Printing Press,Kuwait.
3. Cooper, M. & Johnson. A. (1984). Poisonous Plants in Britain and their effects on Animals and Man. HMSO, England.

4. Chopra, R.N., Nayar, S.L. &Chopra, I.C. (1986). Glossary of Indian Medicinal Plants Council of Scientific and Industrial Research, New Delhi.
5. Department of Biomedical Sciences, Zyed Complex for Herbal Research and Traditional Medicine, Unpublished results.
6. Halliwell B. The antioxidant paradox. Lancet, 2000; 355: 1179-80.
7. B. Auddy, M. Ferreira, F. Blasina, L. Lafon, F. Arredondo, F. Dajas, P.C. Tripathi, T. Seal, B. Mukherjee, Screening of antioxidant activity of three Indian medicinal plants, traditionally used for the management of neurodegenerative diseases, Journal of Ethnopharmacology, 2003; 84,131/138.
8. F. Pourmorad, S. J. Hosseinimehr , N. Shahabimajd, Antioxidant activity, phenol and flavonoid contents of some selected Iranian medicinal plants, African Journal of Biotechnology, 2 June 2006, 5(11): 1142-1145.
9. Y. S. Velioglu, G. Mazza, L. Gao, and B. D. Oomah, Antioxidant Activity and Total Phenolics in Selected Fruits, Vegetables, and Grain Products, Food Research Program, Agriculture and Agri-Food Canada, Pacific Agri-Food Research Centre, Summerland, British Columbia V0H 1Z0, Canada.
10. Jigna PAREKH, Sumitra V. Chanda, In vitro Antimicrobial Activity and Phytochemical Analysis of Some Indian Medicinal Plants, Phytochemical, Pharmacological and Microbiological Laboratory, Department of Biosciences, Saurashtra University, Rajkot 360 005 - INDIA
11. N Prasannabalaji, G Muralitharan , RN Sivanandan , S Kumaran, SR Pugazhvendan, Antibacterial activities of some Indian traditional plant extracts, Asian Pacific Journal of Tropical Disease.
12. J.L. Ríos, M.C. Recio, Medicinal plants and antimicrobial activity, Journal of Ethnopharmacology, 2005; 100: 80–84.
13. Hassan A., Rahman S., Deeba F. and Mahmud S. Antimicrobial activity of some plant extracts having hepatoprotective effects. J. Med. Plants Res., 2009; 3(1): 20-23
14. Mandal P, Sinha Babu SP, Mandal NC. Antimicrobial activity of saponins from Acacia auriculiformis Fitoterapia, 2005; 76: 462-465.